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Occurrence of Novel Groups of the Domain *Bacteria* as Revealed by Analysis of Genetic Material Isolated from an Australian Terrestrial Environment

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A molecular ecological study was performed on an Australian soil sample to unravel a substantial portion of the bacterial diversity. A large fragment of the 16S rRNA gene was amplified, using DNA isolated by lysing the microorganisms directly within the soil matrix, and a clone library was generated. Comparative sequence analysis of 30 clones and dot blot hybridization of 83 additional clones with defined oligonucleotide probes revealed the presence of three major groups of prokaryotes of the domain *Bacteria*. The first one comprises 57 clones that indicate relatives of nitrogen-fixing bacteria of the α-2 subclass of the class *Proteobacteria*; the second group of 7 clones originates from members of the order *Planctomycetales* that, however, reveal no close relationship to any of the described *Planctomycetales* species; 22 clones of the third group are indicative of members of a novel main line of descent, sharing a common ancestry with members of planctomycetes and chlamydiae.

The strategy of polymerase chain reaction (PCR)-mediated amplification of 16S rRNA genes, using either rRNA or rDNA isolated from an environmental habitat, followed by gene cloning, sequencing, and comparative data analysis today provides the most elegant means of obtaining information about the composition of microbial communities (20). This strategy has been used successfully on samples from aquatic environments (4, 9) but has as yet not been tested on samples from terrestrial environments, which have significantly more complex interactions between organic, inorganic, and living material.

Recent results of molecular ecological studies in the marine (3, 9) and thermophilic (27, 31) environments and on symbionts (1) have confirmed the widespread view that the prokaryotic species so far cultured constitute only a very small fraction of the actual microbial population in natural ecosystems (28). Our goal was to use 16S rDNA sequencing and taxon-specific nucleic acid probes to reveal the diversity of prokaryotes in a randomly selected soil sample, a habitat that has as yet not been investigated by these techniques.

MATERIALS AND METHODS

DNA extraction. A subsurface soil sample (depth, 5 through 10 cm) was collected from the Mount Coot-tha region, Brisbane, Queensland, Australia, and processed immediately. Genomic DNA was extracted from the sample by the direct lysis technique (19, 26), with the modification that the hydroxyapatite column chromatography step was replaced by a preparative agarose gel (0.7%) electrophoresis. Fragments larger than 4 kb were recovered by electroelution (22) (Fig. 1).

Amplification and cloning of 16S rDNA. PCR was performed according to Saiki et al. (21), using per reaction 400 ng of purified DNA, 20 nmol of each deoxynucleoside triphosphate, and 100 pmol of each primer, on a DNA thermal cycler (Perkin Elmer Cetus). Amplification was for 32 cycles (annealing for 2 min at 48°C, elongation for 5 min at 72°C, denaturation for 1 min at 94°C). The 5′ and 3′ primers were 5′-GCGGGATCC/GAGTTTGATCCTGGCT CAG-3′ (*Escherichia coli* positions 9 through 27, International Union of Biochemistry nomenclature) and 5′-GGC CGTCGAC/GCCATTGTAGCACGTGTGCA-3′ (positions 1224 through 1243), respectively. The underlined regions represent overhangs with a *Bam*HI and a *Sal*I restriction site, respectively. As judged from the sequence of the target sites, it is unlikely that the primer pair used will allow the amplification of rDNA from archaeas, eukaryas, and representatives of the bacterial phyla defined by the genera *Thermotoga*, *Chloroflexus*, *Thermus*, and *Chlorobium*. PCR products were extracted with chloroform and precipitated with ethanol. Amplified fragments were purified by agarose gel electrophoresis (0.7%) and recovered by using NA-45 paper (22). Digestion with *Bam*HI and *Sal*I, cloning of the 16S rDNA fragments into phage M13 mp19, and preparation of single-stranded DNA of randomly picked recombinants were done according to standard protocols (8, 22).

To sequence the opposite DNA strand, double-stranded 16S rDNA was generated from single-stranded M13 DNA via PCR. Twenty-eight cycles were performed (annealing at 45°C for 2 min, elongation at 72°C for 2 min, denaturation for 1 min at 94°C), using a universal M13 forward primer (5′-GTAAAACGACGGCCAGT-3′) and a universal M13 reverse primer (5′-AACAGCTATGACCATG-3′).

Determination of nucleotide sequences. Analysis of single-stranded DNA with the universal M13 primer was done by using the Taq Dye Primer Cycle Sequencing Kit (ABI, Burwood, Victoria, Australia) and an automatic sequence analyzer (ABI model 370). Sequencing reactions with internal 16S rDNA primers were performed by using Sequenase (U.S. Biochemical, Cleveland, Ohio), and analysis of double-stranded DNA was carried out with the double-stranded DNA Cycle Sequencing System (GIBCO BRL, Glen Waverley, Victoria, Australia).

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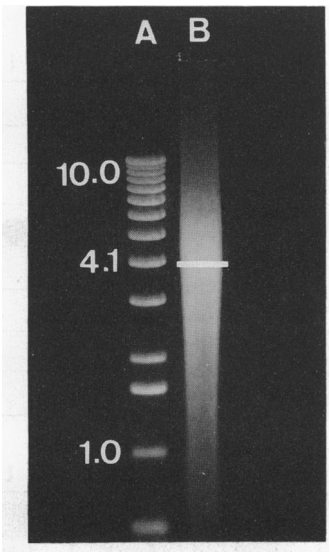

FIG. 1. Agarose gel electrophoresis of purified DNA isolated directly from a soil sample (lane B). Material above the bar was recovered by electroelution and subsequently subjected to PCR-mediated amplification. Lane A is a DNA weight marker (kilobase ladder; GIBCO BRL).

Oligonucleotide synthesis. Oligonucleotide probes and primers were synthesized by using a DNA synthesizer (ABI model 391) and purified according to standard protocols of the manufacturer. Oligonucleotide probes were 5′ labeled with [γ-^{32}P]ATP (12) and purified by using Sephadex G-50 columns according to the instructions of the manufacturer (Pharmacia, North Ryde, New South Wales, Australia).

DNA blotting and Southern hybridization. Single-stranded DNA (250 ng) of M13 clones was blotted onto a nylon membrane (Amersham, North Ryde, New South Wales, Australia), using a dot blot apparatus (Schleicher & Schuell, Dassel, Germany), and treated with UV (260 nm) for 5 min. Prehybridization was done in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–4× Denhardt solution (24) for 1 h at hybridization temperature. Hybridization with a universal clone library probe (γ-^{32}P-labeled 5′ PCR primer; 40 ng, 2×10^6 cpm) in prehybridization solution was carried out at 37°C for 2 h, followed by a washing step for 20 min at 37°C. The accessibility of rDNA was checked by autoradiography. The filter was subsequently rehybridized with two specific probes, one for certain members of the α-2 subclass of the class *Proteobacteria* [5′-GAAAGATTTATCGC-(CT)-GAA-(AG)-GA-3′] and the other for members of the family *Planctomycetaceae* [5′-GGC-(GA)-TGGATTAGGCATGC-3′]. Between each hybridization step, the filter was treated with 0.1× SSC at 75°C and the complete removal of radioactivity was checked autoradiographically. Hybridization and washing steps were performed at 37°C as described above. Following autoradiography, the stringency of the conditions was increased for the hybrids formed with the planctomycete-specific probe by washing at 52°C for 30 min, with renewal of the washing buffer after 15 min.

Phylogenetic analysis. Phylogenetic trees were constructed by the algorithms of Sattath and Tversky (23) and DeSoete (5).

Nucleotide sequence accession numbers. The sequence data have been deposited in the EMBL data base under accession numbers X664373 to X664384.

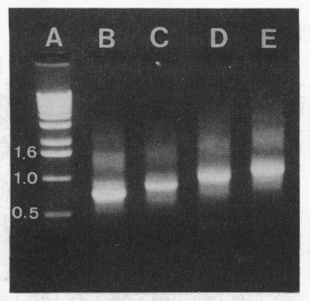

FIG. 2. Agarose gel electrophoresis of PCR-amplified partial 16S rRNA genes, using purified soil DNA (see Fig. 1). Lanes: A, kilobase ladder (GIBCO BRL); B to E, amplification products generated with the universal 5′ primer (see Materials and Methods) and 3′ primers spanning positions 787 to 803 (lane B), 907 to 926 (lane C), 1100 to 1115 (lane D), and 1224 to 1243 (lane E). The latter primer system generated the fragments used to establish a clone library.

RESULTS AND DISCUSSION

Purification of environmental DNA. Prerequisite for molecular approaches to determine the diversity of natural microbial assemblages in environmental samples is the isolation of nucleic acids that are of sufficient purity to perform molecular analyses and represent the actually occurring genetic diversity. For the isolation of nucleic acids from soil samples, two different strategies can be applied: (i) isolation of cells as quantitatively as possible, followed by lysis of the cells and subsequent purification of nucleic acids (cell extraction method), and (ii) lysis of microorganisms within their natural habitats (direct lysis technique). Recovery rates of up to 35% have been reported for the first method (2, 11, 26), but no information is available about whether a representative collection of the community was recovered. We used the second approach, for which a higher chance of obtaining a greater part of the genetic diversity has been suggested (25). The lysis technique applied included a sodium dodecyl sulfate treatment followed by mechanical disruption of the cells with glass beads. PCR-mediated amplification of partial 16S rRNA genes was unsuccessful when the purification steps were restricted to a few phenol-chloroform extractions and a potassium acetate-polyvinylpolyrrolidone treatment. Even a CsCl density gradient centrifugation step did not improve the quality of nucleic acids sufficiently to result in detectable PCR products. DNA of sufficient purity to allow PCR-mediated amplification of rDNA was obtained following a preparative agarose gel electrophoresis (Fig. 1 and 2). It can be assumed that this step removed the amount of interfering substances to an extent that PCR amplification became feasible. Humic acids have been detected in significant amounts in a chemical analysis of this soil sample (unpublished results). The electrophoretic purification step had a second advantage in that small DNA fragments were removed prior to the amplification assay. This step appears necessary to reduce the chance of the formation of chimeric (18) or shuffle (9) products.

Cloning. For the cloning strategy, *Bam*HI and *Sal*I restriction sites were introduced at the 5′ and 3′ ends of the amplified 16S rDNA products, respectively. Both enzymes cut rarely only in 16S rRNA genes (29), e.g., *Bam*HI in certain archaeas, bacteroides, chlorobia, and actinomycetes and *Sal*I in a few archaeas, clostridia, and actinomycetes. Their presence would result in the formation of clones containing fragments that are shorter than the uncleaved

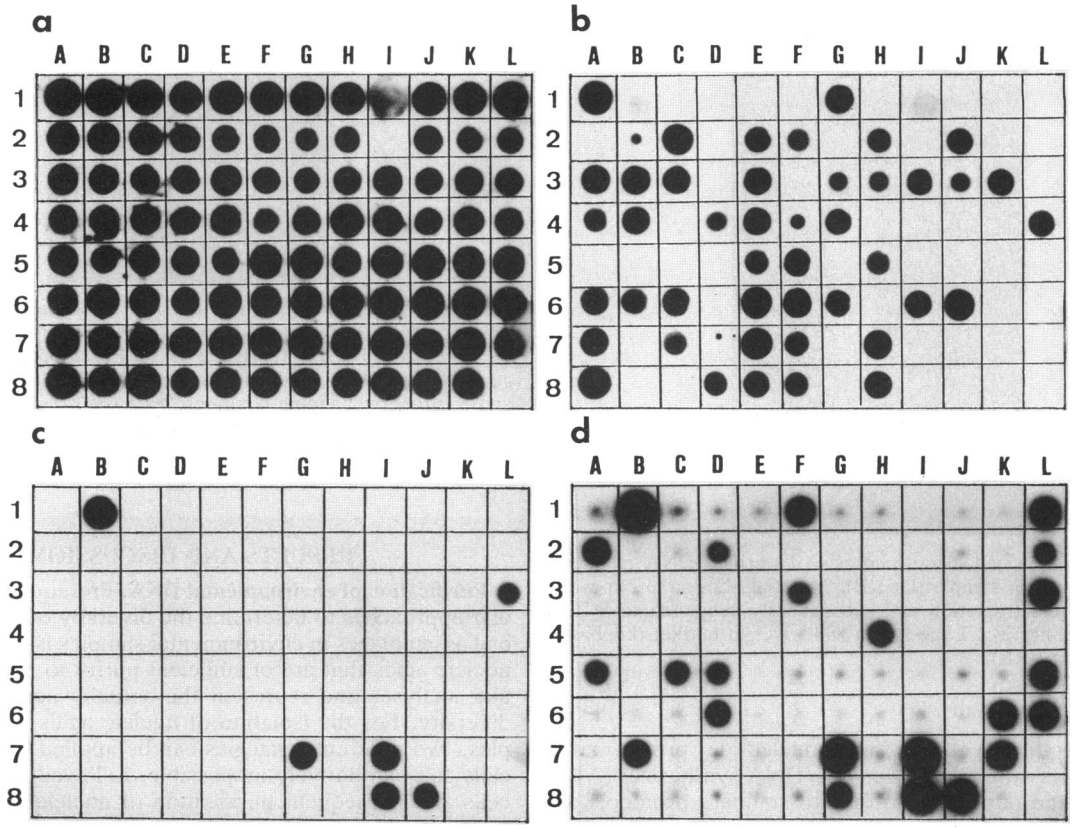

FIG. 3. Dot blot hybridization between isolated partial 16S rDNA clones and oligonucleotide probes of different taxon specificity. The following clones are positive controls: 1A, 1G, and 1I for α-2 proteobacteria; 1B for cluster II; and 1F and 1L for cluster III. The other clones in row 1 are negative controls. The clone at position 2I contains an internal *Bam*HI restriction site and does not hybridize with the universal probe (see Results and Discussion). DNA at position 8L is the M13 mp19 cloning vector. (a) universal clone library probe (5′ PCR primer); (b) α-2 proteobacteria probe; (c) planctomycete probe applied under stringent washing conditions (for cluster II clones); (d) planctomycete probe applied under relaxed washing conditions (for cluster III clones).

amplification products. Depending on the position of the cleavage site, these clones could escape detection with oligonucleotide probes that are used to identify taxon-specific 16S rDNA inserts. A survey of 83 16S rDNA clones by dot blot hybridization, however, demonstrated that a single insert only could not be detected with the 5′ PCR primer used as a universal probe (Fig. 3a). Consequently, only one of these clones contained an internal *Bam*HI restriction site. This clone was the only one that migrated faster in agarose gel electrophoresis than did the other 112 recombinants (not shown). The number of clones with an internal *Sal*I site was not determined. These results indicate that with the cloning strategy used here, the loss of detectable microbial diversity can be considered insignificant.

Data analysis. Initially, about 300 nucleotides from each of 30 cloned partial 16S rDNA genes were sequenced and manually aligned with those of members of all major bacterial lines of descent (13, 32), and the relationship to described species was determined. For selected clones, the determination of the nucleotide sequence was extended to cover ca. 1,000 nucleotides of the 5′ terminus of the molecule (*E. coli* positions 28 to 1023). Phylogenetic analysis was performed on the entire stretch of 1 kb, including the hypervariable regions. Omission of these regions from the analysis led to changes in the branch length but not in the order at which they separate from each other. Application of the algorithms of DeSoete (5) and Sattath and Tversky (23) for constructing least-squares additive trees gave tree topologies so similar that only the results of the latter analysis are presented below.

Phylogenetic relationships. Phylogenetic analysis revealed the presence of two major groups (soil clusters I and II) that fell into one of the established lines of descent, one group (soil cluster III) that grouped separately, and five sequences that could not be convincingly assigned to any described taxa.

The α-2 proteobacteria clones. As reported previously (17), the majority of clones (14 sequences of soil cluster I) revealed a substantial degree of relatedness to nitrogen-fixing members of the α-2 subclass of *Proteobacteria*, e.g., *Bradyrhizobium*, *Azorhizobium*, and "*Photorhizobium*," with the majority of clones forming a new subline of descent of genetically highly similar sequences. The number of representatives of this subclass within the set of 83 clones was evaluated by dot blot hybridization using an α-2 taxon-specific oligonucleotide probe (Fig. 3b); 57 clones (50.4% of the total) could be assigned to this cluster, 43 by hybridization and 14 by sequence analysis.

The planctomycete clones. Seven clones show distinct similarities to sequences of members of the family *Planctomycetaceae*, representing a unique group of the domain *Bacteria* as judged from the available morphological and

TABLE 1. Distribution of signature nucleotides of the 5′ 1,000 nucleotides of the 16S rDNAs of planctomycetes, *C. psittaci*, novel soil prokaryotes (clusters II and III), and members of other main bacterial lines of descent

Position	Nucleotide(s)[a]				
	Planctomycetes (6)	Cluster II (7)	*C. psittaci* (1)	Cluster III (4)	Other *Bacteria* (10)
47–394[b]	G-G	G-G	G-G	G-G	C-G
48[b]	A	A	A	A	Y
50	U	U	G	A	A
52–359[b]	G-C	G-C	G-C	G-C	Y-G
53–358	G-U	G-U	G-U	A-U	A-U
169[c]	G	R	C	U	C
291–309[c]	G-C	R-Y	C-G	U-A	U-A
331	U	U	U	G	G
353	U	U	U	A	A
538[c]	A	A	G	G	u,g
570–88[c]	U-C	U-C	G	G-C	G-C
659[c]	R	R	U	C	U
686[c]	G	G	U	U	U
768[d]	A	A	A	G	A
784–798	G-C	G-C	A-U	G-C	a-u, c-g
772–807[b]	A-U	A-U	A-U	A-U	u-a
811[d]	C	C	C	A	Y
819[d]	A	A	A	G	A
833–853	G-C	R-Y	U-G	U-G	G-C
826–874[d]	C-G	C-G	C-G	U-A	C-G
933[c]	A	A	G	G	G
955[c]	C	C	U	U	U
983:1[c,e]	U	U			

[a] Numbers of analyzed organisms are indicated in parentheses. Y, pyrimidine; R, purine; lowercase letters, present in less than 85% of members of main lines of descent.
[b] Signature, planctomycetes/clusters II and III/chlamydiae.
[c] Signature, planctomycetes/cluster II.
[d] Signature, cluster III.
[e] Colon indicates inserted nucleotide at position 983.

molecular data (7, 14–16). They hybridize with the planctomycete-specific oligonucleotide probe under stringent washing conditions, as shown on six clones in Fig. 3c. Two additional clones that were detected by sequence analysis exhibited the planctomycete-specific nucleotides around position 50 (30, 32) but were chimeric structures (18). The planctomycete clones showed no unusual base pairings or structural deviations other than those reported for members of this phylum. All of them shared the described 16S rRNA signature nucleotides and idiosyncratic features (15, 32) (Table 1) over the stretch of the sequenced 1,000 nucleotides. Phylogenetic analyses (Fig. 4; Table 2) showed that one clone is related to the sequence of *Planctomyces limnophilus* (MC100) and that another is related to *Isosphaera pallida* (MC25), while the remaining clones branch off adjacent to *Gemmata obscuriglobus*. These clones share with *G. obscuriglobus* a unique bulge loop of 13 nucleotides of variable sequence around position 1005 (*E. coli* nomenclature). In comparison with the majority of bacterial 16S rDNAs, the bulge loop between positions 1024 and 1036 is slightly enlarged but not as pronounced as seen in *Thermotoga maritima* (Fig. 5). Another feature of the sequence that supports the relationships of clone MC25 to *I. pallida* and of clones MC1, MC11, MC55, MC98, and MC113 to *G. obscuriglobus* is the relatively high G+C base ratios of their rDNAs (61 and 58.5 to 62.2%, respectively). Similar high values have been reported for the two described species, while the corresponding G+C contents of members of the genera *Planctomyces* and *Pirellula* (54.4 to 55.4%) as well as that of clone MC100 (56.1%) are in the same range as those of neighboring taxa (15), e.g., *Chlamydia* and *Serpulina* sp., a spirochete.

The occurrence of planctomycetes in soil randomly sampled is completely unexpected since all described and uncultivated strains have been isolated and observed in aqueous habitats only. The genera *Gemmata* and *Isosphaera* are each represented by only a single cultivable strain isolated from completely different environments (6, 10). Phylogenetic information of the clones spans the diversity of all four described genera of the family *Planctomycetaceae* which have never been found to thrive in the same habitat. The detection of planctomycete DNA in soil raises doubts about the aquatic environment as the sole habitat for these organisms. The sequence divergence within cluster II clones is significantly higher (71.5 to 82.0% sequence similarity) than that seen among the clones of cluster I, i.e., members of the

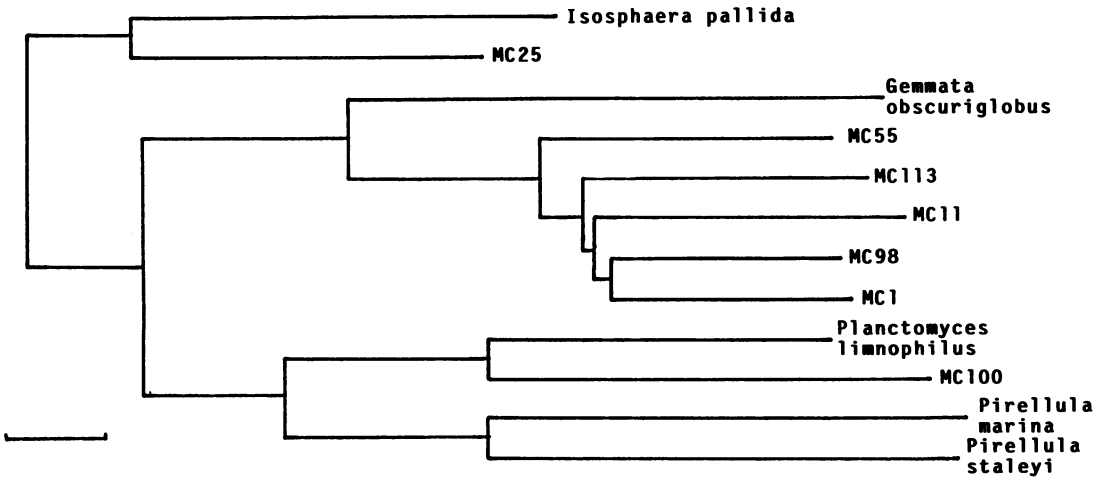

FIG. 4. Phylogenetic relationships of partial 16S rDNA sequences of members of the order *Planctomycetales* and seven clones of soil cluster II. The horizontal components of the branch lengths are proportional to evolutionary distances. The bar represents 20 nucleotides differences.

TABLE 2. Percent similarity between a ca. 1,000-nucleotide-long stretch of the 16S rDNA sequences of soil clone cluster II and representatives of the family *Planctomycetaceae* (above the diagonal space) and equally weighted (Hamming) distances between the 16S rDNA sequences (below the diagonal space)

Species or clone	% Similarity											
	1	2	3	4	5	6	7	8	9	10	11	12
1. *Planctomyces limnophilus*		77.1	80.5	77.6	74.1	85.9	76.5	74.3	73.5	74.1	74.9	72.8
2. *Pirellula marina*	250		82.8	73.1	74.9	74.6	74.6	73.6	72.7	72.5	72.1	72.4
3. *Pirellula staleyi*	218	188		75.0	74.0	77.7	73.8	72.8	72.1	71.5	72.4	70.8
4. *Isosphaera pallida*	245	294	272		82.8	74.5	85.9	75.1	75.5	75.0	74.9	74.6
5. *Gemmata obscuriglobus*	283	274	285	188		71.3	74.0	81.4	80.4	80.7	81.2	80.7
6. MC100	154	278	243	279	314		74.1	73.5	72.4	71.5	82.0	72.4
7. MC25	257	279	286	154	284	261		87.5	87.7	78.0	76.5	77.3
8. MC55	281	289	296	270	203	291	246		89.1	88.5	89.3	87.7
9. MC113	290	298	306	268	214	302	244	119		90.4	89.3	88.8
10. MC1	283	301	311	272	212	312	240	126	105		91.6	88.7
11. MC98	274	305	303	274	205	307	257	117	117	92		90.0
12. MC11	297	302	319	278	213	302	248	135	122	124	110	

α-2 subclass of *Proteobacteria* (>96%). A certain undeterminable degree of divergence can be attributed to the elevated mutation rate of 16S rDNAs that has been observed in culturable planctomycetes, but the selective pressure and the underlying mechanisms still need to be analyzed. The degree of relatedness between clone MC25 and *I. pallida* and between clones MC1, MC11, MC55, MC98, and MC113 on the one side and *G. obscuriglobus* on the other side is so low that the allocation of the organisms that are represented by the clones to the respective genera remains speculative.

Clones of cluster III. Twenty-two clones belong to soil cluster III. All seven clones analyzed by sequencing exhibited high sequence similarities in the 5′-terminal 300 nucleotides of the 16S rDNA. Consequently, only a few clones were further characterized by analysis of additional 700 to 900 nucleotides. While they shared more than 89.9% sequence similarity among each other, values found for these sequences and those of members of other main lines of descent within the domain *Bacteria* were as low (63.0 to 73.4%) as those found between the latter organisms (Table 3). These clones stand phylogenetically isolated and should be considered members of a novel main line of descent within *Bacteria*, branching off within the radiation of a supergroup consisting of the phyla *Bacteroides/Flavobacterium*, *Spirochaetales*, *Planctomycetales*, *Chlamydia*, *Chlorobium/Clathrochloris*, gram-positive bacteria, cyanobacteria, and proteobacteria. Clone cluster III sequences appear to share a common ancestry with members of the phyla *Chlamydia* and *Planctomycetaceae* (Fig. 6). Basically the same position was held when cluster III sequences were compared with a wider selection of bacterial sequences contained in the data base of the NSF Ribosomal RNA Database Project. When analyzed with the algorithm of DeSoete (5), cluster III clones appear slightly more closely related to planctomycetes than to *Chlamydia psittaci*. The relationship between the sequences of cluster III clones, planctomycetes, and *C. psittaci* is supported by the presence of synapomorphic 16S rDNA signature nucleotides (Table 1). It can therefore be predicted that the 16S rDNA and probably other genes of members of clone cluster III are also subjected to an elevated mutation rate. The effect is possibly not as dramatic as in planctomycetes, considering that the several signature nucleotides appear to reflect the "normal" type of isochronically evolving bacteria (32).

The location of several common signature nucleotides around position 50 was used to screen the clone library for the presence of additional cluster III clones with the planctomycete-specific probe under relaxed and stringent washing conditions (Fig. 3c and d). As detected by the difference in positive signals between these two steps, 15 clones were identified as belonging to cluster III.

The degree of divergence between the sequences of clones of soil cluster III is significantly lower than that of the planctomycete clones. As judged from the similarity value that separates the most divergent clones of cluster III (90%), the four clones could be considered individual species within one or two new higher taxa. A decision about the ranks and formal description, however, must await the availability of phenotypic data.

Of the 113 clones analyzed, 86 clones and 2 chimeric

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E. coli           UC-CACGG-AA----------GUUUUCAGAGAUGAGAAUGUGCC------UUCG----GGAACCGUGAGA
S. innocens       UGUAAGAUGAA----------UGAUUUAGAGAUAAGUCAAACC-------GCAA----GGACGUUUUACA
C. psittaci       UG-UAUUU-GA----------CCGCGGCAGAAAUGUCGUUUUCC------GCAA-----GGACAGAUACA
clone MC18        UGCAUCUCUAA----------GCUGGUGAAAGCCAGUGACUCCC-----GAAA---GGGAGAAUUUGCA
Pl. limnophilus   UGGUGGGAUUA----------GUUGGCUGAAAGGUCAAUGACGCC-----UUCG---GGUGGAACCAUCA
Pl. maris         UGCUUGUAUUA----------GCUCUGUGAAAGCAGAGUGACGCC-----UUCG---GGUGGAACUUGCA
P. marina         UGCUUAGG-AA----------UCUUCCUGAAAGGGAGGAGUGCUCU----GCA-----AGAGGCCUUUCA
P. staleyi        UGCUUAGG-AA----------UCUUCCUGAAAGGGAGGAGUGCUC-----GCAA-----GAGGCCUUUCA
G. obscuriglobus  UGUGCGA--AAGCGCUCGGCAGUAGGACCCGGAAACGGAACGGACCCCA----GCAA-UGGGGGGACCGAGCA
clone MC113       UGUCCGA--AAGGGGGAGGAAGUACCCUGCGGAAACGUAAGGGGAACGGUAC-CCA--GCCCGGACCCUCCCA
clone MC98        UGUGCGA--AAGCGGUAGCAAGUAGGGUGCGGAAACGUUCCCCCAACGGUAU-CCA--GUCCGGAAGCUACUA
clone MC55        UGUGCGA--AAGCGGCAGGGAAUAGCCCGCGGAAACGUGGGGCCAACGGUAU-CCA--GUCCGGAGCCUGCCA
clone MC11        UGUGCGA--AAGCGCAGGGCAGUAGGGCGCGGAAACGUGUCCCCAACGGUAC-CCA--GUCCGGAACCCUgUA
clone MC1         UGUGCGA--AAGCGGUGGGGAGUAGGGUGCGGAAACGUUACCCCAACGGUAC-CCA--GUCCGGAACCCACCA
T. maritima       UG-CCGGU-GG----------UACCUCCCCGAAAGGGGUAGGGACCCAGUCCUUCGGGACUGGGAGCCGGCA
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FIG. 5. Comparison of sequences of the variable 16S rDNA region from positions 997 to 1044 between *G. obscuriglobus* and related soil clones, other planctomycetes, and representatives of the domain *Bacteria*. *S. innocens*, *Serpulina innocens*. For other genus names not given in the text, see Tables 2 and 3.

TABLE 3. Percent similarity between a ca. 1,000-nucleotide-long stretch of the 16S rDNA sequences of soil clone cluster III and representatives of several main lines of descent of the domain *Bacteria* (above the diagonal space) and equally weighted (Hamming) distances between the 16S rDNA sequences (below the diagonal space)

Species or clone	Phylum	% Similarity													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *Escherichia coli*	Proteobacteria		75.0	74.8	66.8	69.2	66.9	70.5	70.0	70.7	70.6	70.5	69.7	69.0	70.8
2. *Anacystis nidulans*	Cyanobacteria	287		75.1	64.8	66.8	69.9	70.4	70.3	70.4	70.4	70.4	67.9	68.2	70.6
3. *Streptomyces lividans*	Gram-positive bacteria	280	277		66.1	70.3	72.0	73.4	72.1	73.0	73.0	70.9	69.9	69.1	72.2
4. *Clathrochloris sulfurica*	Green sulfur bacteria	367	389	375		65.5	64.3	63.0	64.8	64.5	64.5	64.0	62.2	61.4	64.8
5. *Bacteroides fragilis*	*Bacteroides/Flavobacterium*	341	367	340	380		66.3	66.9	66.3	66.8	66.8	66.7	65.1	65.1	64.5
6. *Serpulina* sp.	*Spirochaetales*	365	334	311	393	372		70.5	70.3	70.2	70.1	67.7	67.5	68.0	68.1
7. MC31	New phylum	327	338	295	408	365	327		89.9	89.8	90.0	70.9	69.4	70.8	69.8
8. MC17	New phylum	332	340	307	388	371	329	117		94.8	83.8	70.2	70.9	71.2	70.9
9. MC15	New phylum	325	338	300	391	367	330	119	65		97.5	70.7	70.2	70.7	69.5
10. MC18	New phylum	326	339	299	390	368	331	116	75	36		70.4	70.1	70.4	69.5
11. *Chlamydia psittaci*	*Chlamydia*	327	339	322	397	369	357	322	330	325	328		70.1	69.5	68.5
12. *Planctomyces limnophilus*	*Planctomycetales*	335	355	334	416	385	359	339	322	330	331	331		80.5	70.4
13. *Pirellula staleyi*	*Planctomycetales*	343	351	342	425	386	354	324	317	326	328	337	218		68.1
14. *Thermotoga maritima*	*Thermotogales*	323	326	308	389	392	352	335	321	336	337	348	328	353	

clones could be assigned either to described taxa or to a novel taxon by sequence analysis or dot blot hybridization. Five of the remaining 25 clones were sequenced but could not be convincingly identified through phylogenetic analysis. Sequence information is not available for the other 20 clones that did not react with the cluster-specific oligonucleotide probes.

None of the organisms for which sequences have been obtained from the partial 16S rDNA clone library have been successfully cultivated. The phenotype, and consequently the composition, of enrichment media and cultivation conditions cannot be predicted per se from the phylogenetic relatedness between uncultured and cultured strains. Even within the family *Planctomycetaceae*, growth requirements vary significantly, i.e., for *I. pallida* and *G. obscuriglobus* and even more so between planctomycetes and chlamydiae. Assuming that the DNA isolated from the environmental sample originated from bacteria that are or were part of the microbial community, the genetic diversity of the 16S rDNA clones of soil group II and III members will allow us to

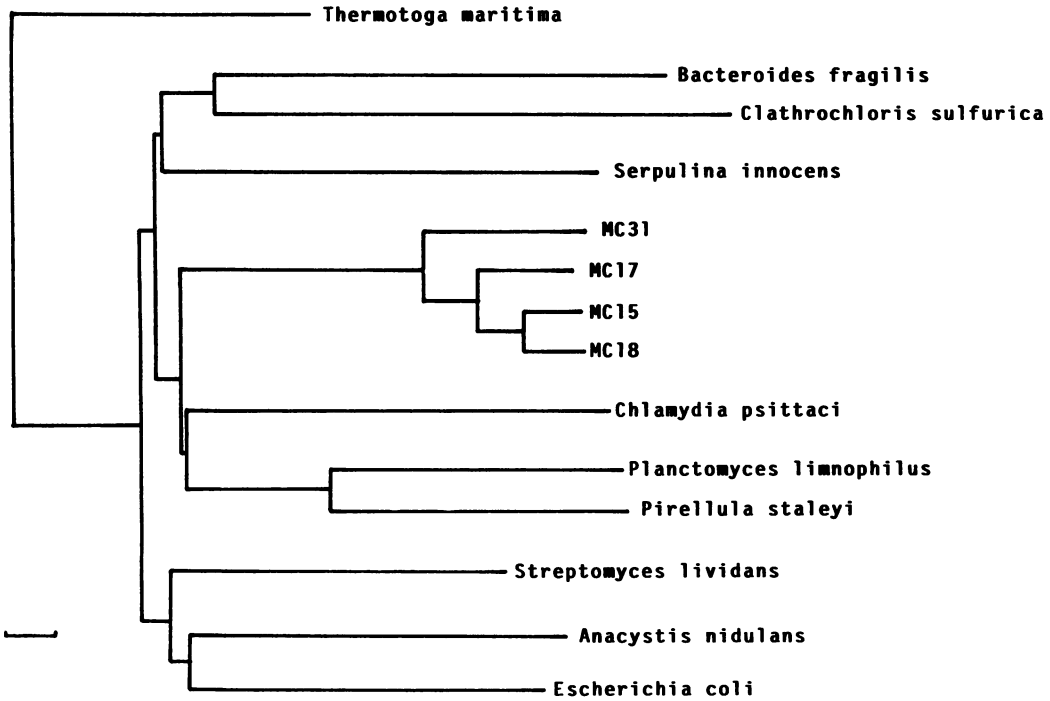

FIG. 6. Phylogenetic relationships of partial 16S rDNA sequences of four clones of soil cluster III and representatives of several main lines of descent of the domain *Bacteria*. The horizontal components of the branch lengths are proportional to evolutionary distances. The bar represents 20 nucleotides differences.

identify the relevant strains in situ and to investigate the distribution and diversity of the novel types of organisms in time and space with nucleic acid probes.

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